PROVINCIAL GUIDELINES AND LABORATORY PROCEDURES FOR MEASURING ACUTE LETHAL TOXICITY OF LIQUID EFFLUENTS TO FISH

Province of
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Ministry of
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PROVINCIAL GUIDELINES AND LABORATORY PROCEDURES FOR MEASURING ACUTE LETHAL TOXICITY OF LIQUID EFFLUENTS TO FISH

Report of the Bioassay Task Force

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Province of
British Columbia

MEMORANDUM

To: R. H. Ferguson,
Director,
Waste Management Branch.

Date: November 19, 1982.

File: SU 64.1205

Re: Standardization of Bioassay Procedures

Please find enclosed the final report of the bioassay task force. The report presents guidelines and laboratory procedures for fish bioassays. These procedures, which are patterned on the federal procedures, should produce consistent and comparable results for any one type of test measuring acute toxicity of effluents to fish. The document, therefore, meets the requirements of the Pollution Control Board which recommended the publication of the guidelines.

The selection of sample and bioassay type was studied by the task force and draft guidelines were produced. However, after reviewing comments from several agencies and organizations the task force decided that much more effort would be needed to produce acceptable guidelines, and therefore none were included in this report. Also, the subject of other aquatic bioassay methods, such as in situ tests, sub-lethal tests and tests employing organisms other than fish, was considered to be too far ranging to be included in the terms of reference. Any of these topics could be studied in future inquiries if you think that provincial guidelines are needed.

R. J. Buchanan, Ph.D.,
Water Management Branch.

Encl.

MEMBERS OF THE BIOASSAY TASK FORCE

R.J. Rocchini (Chairman)	Aquatic Studies Branch, Ministry of Environment
J.A. Servizi	International Pacific Salmon Fisheries Commission
R.G. Watts	Environmental Protection Service, Environment Canada
S. Horvath	Environmental Laboratory, Ministry of Environment
M.J.R. Clark	Waste Management Branch, Ministry of Environment
H.J. Singleton	Aquatic Studies Branch, Ministry of Environment
R.H. Young	MacMillan Bloedel Ltd., for Council of Forest Industries of B.C.
A. Sholund	Imperial Oil Ltd., for British Columbia Petroleum Association
A.J. Jordan	Rescan Environmental Services Ltd., for Mining Association of British Columbia
D.J. McLeay	D. McLeay and Associates Ltd., for Council of Forest Industries of B.C., British Columbia Petroleum Association and Mining Association of British Columbia

SUMMARY

A task force was formed from government agencies and industry to draft guidelines and uniform laboratory procedures for fish bioassays throughout the Province. Use of these procedures and guidelines should produce consistent results for any one type of test used to monitor the acute toxicity of effluents to fish.

Procedures are given for holding and acclimating fish, transporting and storing effluent samples and conducting fish bioassays.

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1.0 INTRODUCTION

Following a public hearing in February, 1980, on pollution in the lower Fraser River, the Pollution Control Board concluded that guidelines and laboratory procedures for fish bioassays be established. Subsequently the Board recommended that the Waste Management Branch publish such guidelines and laboratory procedures, after consultation with interested parties.

The recommendation came about because certain results on acute toxicity of municipal effluents were not readily comparable due in part to variations in test procedures.

There are many methods of measuring acute toxicity, all of which have their application, but the use of similar laboratory procedures for fish bioassays throughout the Province will permit comparison of results for any one type of test. The purpose of the bioassay tests detailed here is to monitor the acute lethal toxicity of effluents to fish; that is to determine if diluted or undiluted effluent samples, tested under controlled laboratory conditions, cause fish mortalities.

The Waste Management Branch set up a task force with representatives from the Federal and Provincial Government and industry. The general terms of reference of the task force were as follows:

- To establish provincial guidelines and laboratory procedures for measuring the acute lethal toxicity of liquid effluents to fish.
- To specify effluent handling and sampling procedures.

In developing these procedures an attempt was made to balance scientific and practical considerations and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be used.

Several aquatic bioassay methods are not included in this report as they are outside the terms of reference. These include, for example, *in situ* tests, sub-lethal tests and various tests employing organisms other than fish, such as algae, daphnia, or bioluminescent bacteria.

The report describes methods of effluent sampling and types of bioassays that can be performed, but it does not cover the selection of suitable sample and bioassay type for a given effluent. The selection of sample and bioassay type will depend on many factors, including the stability of the effluent and the variability of effluent toxicity. Also important are volume of effluent discharged, dilution in the receiving water, potential effects on water quality and aquatic life, cost of sampling and testing and site location and accessibility. Consideration of these factors could, if required, be the subject of a separate inquiry.

2. GENERAL PROCEDURES FOR FISH BIOASSAYS

General procedures for fish bioassays are based on the federal report: Standard Procedure for Testing the Acute Lethality of Liquid Effluents (1980). Some changes were made to the federal procedures to suit British Columbia conditions. It is believed that the use of the procedures given here will allow consistent and comparable bioassay results to be obtained for any one type of test.

This section lists laboratory test conditions which should be followed for all fish bioassays, whether static or flow-through. The listing is followed by a number of explanatory notes, which are intended to clarify certain points of the test conditions.

2.1 TEST CONDITIONS

2.1.1 Subject to item 2.1.14, rainbow trout (Salmo gairdneri) are to be used as the test species.

2.1.2 Only healthy stocks of fish acclimated as described in section 2.2.1, are to be used. Individual fish may be tested only once.

2.1.3 Individual fish are to have been actively feeding for a minimum of two weeks, and are to weigh between 0.2 and 5 grams. The length of the largest fish should not be more than two times the smallest in the same test.

2.1.4 A minimum of ten test fish are to be exposed to each test solution for a given time. At least ten control fish are to be exposed concurrently to the control water for at least the same time period.

2.1.5 The test is rendered invalid if fish mortality exceeds 10 percent in the control water.

2.1.6 For every one gram of fish, there should be at least 0.5 litre of test solution or control water for every 24 hours that the fish are exposed.

2.1.7 The minimum water depth in any test vessel should be 15 cm.

2.1.8 The test should be conducted at 15 ± 1°C.

2.1.9 Prior to the addition of fish, the control water and each test solution are to be pre-aerated, using a disposable glass pipette (not an airstone) in each test vessel, at no more than 7.5 mL $min^{-1}L^{-1}$, for not more than 120 minutes, to raise the dissolved oxygen to at least 60 percent saturation.

2.1.10 The test shall begin either after achieving at least 60 percent dissolved oxygen saturation within the maximum time limit (120 minutes) for aeration, or immediately after the maximum time limit is reached should 60 percent dissolved oxygen saturation not be achieved. If 60 percent dissolved oxygen saturation is not reached, the dissolved oxygen shall be measured at least twice in each 24-hour period during the test. Low dissolved oxygen may cause stress and contribute to observed mortalities.

2.1.11 An aeration rate of no more than 7.5 mL $min^{-1}L^{-1}$ can be applied to each test solution and control water throughout the test period.

2.1.12 Exclusive of aeration, no food or chemicals should be added to the test solutions or control water, except for pH neutralization if specified for compliance testing.

2.1.13 The total number of dead fish should be recorded for each test solution at least once each day, up to the completion of the test. Dead fish should be removed at least once each day. Fish are considered dead when they fail to respond to gentle prodding.

2.1.14 Effluents containing only freshwater or having a salinity of less than or equal to 10 parts per thousand whether discharged into freshwater or seawater should be tested with rainbow trout acclimated to freshwater. Effluents containing seawater and/or brine and having a salinity greater than 10 parts per thousand and deposited into seawater should be tested with an appropriate fish species acclimated to seawater of similar salinity to that of the effluent. Effluents containing brine and deposited into freshwater should be tested with rainbow trout acclimated to freshwater.

2.1.15 When the effluent sample must be transported or stored, the sample should be kept in sealed non-toxic containers excluding any air. The sample should be stored in darkness and tested as soon as possible, but no more than five days after collection. For effluent sample types shown to be inherently unstable, a shorter time between collection and testing is advised and should be specified for compliance testing. Alternatively, on-site testing may be considered. To evaluate effluent stability the results of static tests performed on a grab sample immediately after collection and after four days of storage should be compared. If after four days the toxicity, expressed in toxic units*, changes by 50 percent or more, the effluent could be classed as highly unstable.

2.1.16 The effluent sample may be kept at ambient temperature for 48 hours after collection, except in the case of extreme ambient temperature during transport and storage. Samples should be maintained at about 4°C thereafter, except for those portions adjusted to the bioassay temperature within 24 hours of testing. For effluent sample types shown to be inherently unstable, as described in section 2.1.15, temperature control may be required immediately after collection, and should be specified for compliance testing. Alternatively, on-site testing may be considered.

2.2 EXPLANATORY NOTES

2.2.1 HOLDING AND ACCLIMATION OF FISH

Fish should be acclimated to the following laboratory conditions over a period of at least two weeks prior to testing. The purpose of acclimation

$$*\text{toxic units} = \frac{100}{\text{96h LC50}}$$, where 96h LC50 is in volume percent

(Esvelt *et al.*, 1973).

is to determine that fish are healthy and to allow them to adjust to the holding conditions. A record should be kept of water quality, fish disease and mortality during acclimation and holding.

a) Water Quality

Holding, diluent and control water can be receiving water, or water from another source having alkalinity, hardness and nonfilterable residue no greater than the annual mean in the receiving water. It is recommended that water quality be monitored according to the characteristics and frequency in Table 1.

b) Holding Facilities

The acclimation tanks and accessories must be made of non-toxic materials, such as glass, porcelain, fibreglass, stainless steel, polyethylene, acrylic, polypropylene or fibreglass-reinforced polyester. Copper, galvanized material, natural rubber, brass and lead must not come in contact with holding, acclimation or dilution water, or with effluent samples or test solutions. The acclimation tanks should be located away from any physical disturbances and preferably in a location separate from the test vessels.

During acclimation, a constant flow of water through the holding tanks is necessary. Rates of water exchange should be a minimum of 1.4L/g of fish per day. To prevent overcrowding there should be at least one litre of water for every 10 g of fish in the holding tank (Sprague, 1973).

TABLE 1

RECOMMENDED PARAMETERS AND SAMPLING FREQUENCY FOR DILUENT AND CONTROL WATERS

Parameter	Frequency		
	Monthly	Twice Yearly (Spring and Fall)	Source Dependent
Total ammonia nitrogen	✓		
Specific conductance	✓		
Dissolved oxygen	✓		
Hardness	✓		
Nonfilterable residue	✓		
pH	✓		
Residual chlorine			✓
Salinity			✓
Temperature	✓		
Total alkalinity	✓		
Total organic carbon	✓		
Total organophosphorus pesticides			✓
Total organophosphorus pesticides plus polychlorinated biphenyls			✓
Total: aluminum, arsenic, calcium, cadmium, cobalt, chromium, copper, iron, mercury, potassium, magnesium, manganese, sodium, nickel, lead and zinc		✓	

c) Photoperiod

The photoperiod for acclimation and testing should be either a constant sequence of 14 hours of light and 10 hours of darkness or the natural photoperiod. It is recommended that lights be turned on or off gradually over at least 15 minutes.

d) Aeration in Holding Tanks

Supplementary aeration by filtered, oil-free compressed air should be provided if necessary to keep the dissolved oxygen level at greater than 80 percent saturation.

e) Cleaning of Holding Tanks

Holding tanks should be kept clean. Designs for tanks which are partially self-cleaning are available. However, periodic siphoning of settled material is usually necessary. Tanks should also be disinfected between batches of fish to minimize the occurrence of disease.

f) Temperature

Since the temperature of the holding water used during the transfer of fish from the hatchery to the laboratory may be outside of the acceptable limits for the test (15 ± 1°C), it may be necessary to gradually change the temperature until the desired temperature for acclimation is achieved. The fish should be held at this temperature for a 14-day acclimation period before being used in a bioassay.

g) Feeding

Fish shall be fed a recognized commercial fish food suitable for rainbow trout. The mean condition factor K for rainbow trout ($K = \frac{100w}{\ell^3}$) at the time of testing shall fall in the range 0.7 to 1.3, where w is the wet weight in grams and ℓ the fork length in centimetres. Fish shall not be fed for at least 12 hours prior to testing.

h) Disease Detection and Control

Daily inspection of fish in holding tanks is an essential part of the detection of disease, as "healthy" versus "unhealthy" conditions should be recognized (Roberts and Shephard, 1974). Fish should be disease-free, and observed mortalities during acclimation and holding should be less than 1 percent per week.

2.2.2 TEST AND CONTROL VESSELS

Glass is the preferred material for test and control vessels. Vessels, as well as measurement devices shall be thoroughly cleaned and rinsed in accordance with good laboratory practice.

2.2.3 START OF TEST

Prior to commencing the bioassay the test vessels and accompanying tubing should be rinsed with control water. Test concentrations for 96h LC50 determinations may be an appropriate series of five or more. To facilitate the calculation of bioassay results, for example the interpolated values for LC50 tests, it is recommended that a test concentration series be chosen from a logarithmic table such as Table 2. The aliquots of effluent used in the test must be representative of the total effluent sample available. The aliquots must be thoroughly mixed immediately before introduction into the test vessels. Temperature, dissolved oxygen levels and pH should be recorded for each test vessel and the fish randomly introduced.

2.2.4 SPECIAL CONSIDERATIONS FOR CERTAIN TYPES OF SAMPLE

If the effluent sample contains a high level of settleable solids, these should be re-dispersed throughout the test. The re-circulation of diluted or undiluted suspensions through test vessels with vertical sides and steeply sloped conical-shaped bottoms is recommended for this purpose (Noggle, 1978). Samples in the collection containers must be agitated thoroughly just prior to pouring to ensure the re-suspension of all solids.

TABLE 2

SERIES OF EFFLUENT CONCENTRATIONS FOR USE IN 96h LC50 TESTS

Column (Number of Concentrations Between 10 and 1)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
3.2	4.6	5.6	6.3	6.8	7.2	7.5	7.7	7.9	8.1	8.2	8.4	8.5	8.6	8.7
1.0	2.2	3.2	4.0	4.6	5.2	5.6	6.0	6.3	6.6	6.8	7.0	7.2	7.3	7.5
	1.0	1.8	2.5	3.2	3.7	4.2	4.6	5.0	5.3	5.6	5.9	6.1	6.3	6.5
		1.0	1.6	2.2	2.7	3.2	3.6	4.0	4.3	4.6	4.9	5.2	5.4	5.6
			1.0	1.5	1.9	2.4	2.8	3.2	3.5	3.8	4.1	4.4	4.6	4.9
				1.0	1.4	1.8	2.1	2.5	2.8	3.2	3.5	3.7	4.0	4.2
					1.0	1.3	1.7	2.0	2.3	2.6	2.9	3.2	3.4	3.6
						1.0	1.3	1.6	1.9	2.1	2.4	2.7	2.9	3.2
							1.0	1.3	1.5	1.8	2.0	2.3	2.5	2.7
								1.0	1.2	1.5	1.7	1.9	2.2	2.4
									1.0	1.2	1.4	1.6	1.8	2.0
										1.0	1.2	1.4	1.6	1.8
											1.0	1.2	1.4	1.5
												1.0	1.2	1.3
													1.0	1.2
														1.0

Midpoints between Concentrations in Column X will be found in Column (2X+1).

The listed values can represent concentrations expressed as percent by volume or weight, or milligrams per litre or parts per million (ppm) by weight or volume.

Values may be multiplied or divided, as necessary, by any power of 10.

An example of table use and subsequent LC50 determination is as follows:-

A toxic sample was estimated to have a 96h LC50 value near 1% concentration by volume. From Column 4, the following series of five concentrations were selected to include and bracket the estimated LC50 value; 2.5%, 1.6%, 1.0%, 0.63% and 0.4% (the last two values were Column 4 listings divided by 10). The bioassay provided the following observations of cumulative fish mortality after 96 hours of exposure; 100% mortality in concentrations of 2.5%, 1.6% and 1.0%, 0% mortality in concentrations of 0.63% and 0.4%. The resulting 96h LC50 value of 0.79% was determined from column 9 (2x column 4+1) of the table and confirmed by a semilogarithmic plot of the % concentration versus the % mortality data.

For further information on the use of such a table see the Environment Laboratory Manual, 1979.

Test suspensions should be continuously drawn from the bottom of the cone and pumped onto the surface of the suspension. Fish within these test vessels are to be held in soft mesh baskets for protection from pumps and to permit daily inspection for mortalities. The level of suspended solids in each test vessel should be measured upon introduction of fish, and at 24-hour intervals thereafter.

If the effluent sample contains a high level of floatable material (ie. oil or surfactants), test suspensions should be agitated throughout the test to ensure mixing and exposure of fish to soluble constituents. The re-circulating conical vessels described above may be used for this purpose. Alternatively, cylindrical test vessels with individual impellers should be used to maintain homogeneous emulsions (EPS 1973, Blackman et al., 1978). Fish must be protected from impellers by rigid-frame cages.

For all highly colored or opaque effluent samples, or for samples producing foam in the test vessel, fish are to be inspected at least daily by raising them to the surface in a suitable basket, or by carefully drawing a dip net through the vessel.

2.2.5 OTHER OBSERVATIONS

Measurements should be made daily of the aeration rate, temperature, pH and dissolved oxygen of each bioassay test solution and of the control water.

2.2.6 BIOASSAY RECORD SHEETS

The information shown in Table 3 should be recorded on bioassay record sheets.

TABLE 3

INFORMATION TO BE RECORDED ON BIOASSAY INFORMATION RECORD SHEETS

A. Laboratory details:
- Laboratory identification
- Tester(s)

B. Sample details:
- Sample type (grab, composite, on-line)
- Sample source
- Sample description
- Collection date and time
- Storage/shipping details
- Date and time received in laboratory

C. Control/Diluent Water details:
- Water type
- Water source (date, time collected if not tap water)
- Current values for parameters listed in TABLE 1.

D. Test Organism details:
- Species
- Source
- Treatments
- Acclimation details
- Food type and quantity fed
- Feeding schedule
- Average length, weight, and condition factor, with ranges
- Holding temperature
- Photoperiod

E. Test details:

- Test type performed (special apparatus if used)
- Exposure duration
- Exposure dates
- Photoperiod
- Aeration details, preaeration details
- Replacement/flow rate
- Test temperature
- Test depth
- Test volume, test vessel description
- Number of test organisms per test vessel
- Loading rate/day
- Concentrations established (w/w, v/v, active ingredient)

F. Test Observation details:

- Effects, mortalities/test concentration/observation time
- pH, D.O., temperature/test concentration/observation time

G. Test Result details:

- Limit/LT50/LC50 determined value with method
- 95% confidence limits & statistical procedures used

H. Other Appropriate details.

3. SPECIFIC PROCEDURES FOR FISH BIOASSAYS

This section defines different bioassay procedures recommended for measuring the acute lethal toxicity of effluents to fish. The test conditions described in section 2 apply to all these procedures. This section also defines the types of effluent sample which may be used for bioassays and the measurements used to express acute lethal toxicity.

3.1 DESCRIPTION OF SPECIFIC BIOASSAY PROCEDURES

3.1.1 STATIC BIOASSAY

In the static technique, the fish are exposed to the same test and control solutions for the duration of the test. This test may employ either grab or composite effluent samples as described in section 3.2.

3.1.2 STATIC REPLACEMENT BIOASSAY

In the static replacement technique, test fish are exposed to fresh test solution every 24 hours for the duration of the test by replacing the test solution. Control fish are treated similarly.

The replacement of solution may be carried out either by transferring the fish from one test vessel to another, or by renewing at least 90 percent of the test solution in the original test vessel. Control water is replaced similarly. This test may employ either grab or composite effluent samples as described in section 3.2.

3.1.3 FLOW THROUGH BIOASSAY

In the flow through technique, test solution flows continually through the test vessel, at a rate of at least 2 litres per gram of fish per day for the duration of the test. To ensure adequate replacement of solution

the total flow during any six-hour period should be equal to or greater than the volume of test solution in the test vessel. Control fish are subject to the same conditions using control water.

This test may employ either a grab or a composite effluent sample as described in section 3.2. The sample flows continually from a storage tank using metering and dilution equipment of a type such as described in Standard Methods (1980).

3.1.4 ON-LINE BIOASSAY

In this technique, test solution flows continually through the test vessel in the manner and at the rate described for the flow through bioassay (section 3.1.3). However, rather than using a grab or composite effluent sample, effluent is conveyed directly and continuously from the effluent source to the metering and dilution system and hence to the test vessel. This test must therefore be performed at the site where the effluent originates.

3.2 DESCRIPTION OF EFFLUENT SAMPLE TYPES

3.2.1 GRAB SAMPLE

The grab sample is a single or discrete sample of the effluent collected in a period not exceeding 15 minutes where practical. In some cases sequential grab samples may be specified to supply effluent for static replacement or flow through bioassays.

3.2.2 COMPOSITE SAMPLE

a) Simple Composite Sample

The simple composite sample is composed of a series of equal aliquots of effluent collected at least hourly over a total time period of 24 hours

or as related to the periodicity of the effluent discharge. The collection of the aliquots may be intermittent or continuous. Continuous collection requires a constant flow of effluent to the sample containers. The total series of aliquots collected comprising a single composite must be thoroughly mixed in a single container before use. In some cases simple composite samples may be collected sequentially to supply effluent for static replacement or flow through bioassays.

b) Flow Proportional Composite Sample

The flow proportional composite sample is composed of a series of aliquots of effluent collected at intervals of time so that the rate at which each aliquot is collected is proportional to the rate at which the effluent is being discharged. The total collection time should be 24 hours or as related to the periodicity of the effluent discharge. The collection of the aliquots may be continuous or intermittent. Continuous collection requires a continuous flow of effluent to the sample containers. For intermittent collection aliquots must be collected at least once per hour. The total series of aliquots collected comprising a single composite must be thoroughly mixed in a single container before use. In some cases flow proportional composite samples may be collected sequentially to supply effluent for static replacement or flow through bioassays.

3.3 DESCRIPTION OF MEASUREMENTS FOR EXPRESSING ACUTE LETHAL TOXICITY

3.3.1 LIMIT BIOASSAY

The limit bioassay is used to determine if the toxicity of a sample is above or below a specified value. This test requires the survival of 50 percent or more of the test fish over 96 hours, in an effluent concentration equal to the specified value. The test may therefore require the use of only one effluent concentration plus a control.

3.3.2 LT50

The LT50 is the calculated lethal time to death of 50 percent of the test fish at a specified concentration of the effluent. The LT50 test should be continued for 96 hours for surviving fish. The 95 percent confidence limits for the LT50 values should be calculated.

3.3.3 96h LC50

The 96h LC50 is the calculated concentration of effluent which is lethal to 50 percent of the test fish during a 96-hour exposure. In setting up the 96h LC50, at least five different concentrations of effluent in dilution water plus a control should be used. To calculate the 96h LC50 from the test data, the methods described by Standard Methods (1980) may be used. The 95 percent confidence limits should be calculated for 96h LC50 values.

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